CARBOHYDRATE RESEARCH

The structure of the exopolysaccharide of *Pseudomonas fluorescens* strain H13 [1]

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Carbohydrate Research (ISSN 0008-6215) — For 1997 volumes 297–306 are scheduled for the period January–December 1997. Annual Institutional Subscription Rates 1997: Europe, The CIS and Japan 7582 Dutch Guilders. All other countries US$4680. Dutch Guilder prices exclude VAT. Non-VAT registered customers in the European Community will be charged the appropriate VAT in addition to the price listed. Prices include postage and insurance and are subject to change without notice. Any enquiries relating to subscriptions should be sent to: The Americas: Elsevier Science Customer Support Department, P.O. Box 945, New York, NY 10010, U.S.A. [Tel: (+1) 212-633-3730/1-888 4ES-INFO. Fax: (+1) 212-633-3680. E-mail: usinfo-f@elsevier.com]. Japan: Elsevier Science Customer Support Department, 9-15 Higashi-Azabu 1-chome, Minato-ku, Tokyo 106, Japan [Tel: (+3) 5561-5033. Fax: (+3) 5561-5047. E-mail: kyf04035@niftyserve.or.jp]. Asia Pacific (excluding Japan): Elsevier Science (Singapore) Pte Ltd, No. 1 Temasek Avenue, 17-01 Millennia Tower, Singapore 039192 [Tel: (+65) 434-3727. Fax (+65) 337-2230. E-mail: asianinfo@elsevier.com.sg]. Rest of the world: Elsevier Science Customer Service Department, P.O. Box 211, 1001 AE Amsterdam, The Netherlands [Tel: (+31) 20-485-3757. Fax: (+31) 2-485-3432. E-mail: nlinfo-f@elsevier.nl].

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The structure of the exopolysaccharide of *Pseudomonas fluorescens* strain H13 [1]

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Abstract

An acidic exopolysaccharide was isolated from *P. fluorescens* strain H13. The structure of the polysaccharide repeating unit was determined using chemical methods and 1D and 2D NMR techniques. The repeating unit was characterized as a trisaccharide composed of D-glucose, 2-acetamido-2-deoxy-D-glucose and 4-*O*-acetyl-2-acetamido-2-deoxy-D-mannuronic acid.

Keywords: Exopolysaccharide; *Pseudomonas fluorescens*; Structure; NMR spectroscopy

1. Introduction

The structures of several exopolysaccharides (EPSs) produced by strains of *P. fluorescens (marginalis)* have been characterized in this laboratory [1] as an integral part of a program to determine the commercial potential of polysaccharides derived from plant-associated bacteria. Whether due to the presence of uronic acids or substituents such as pyruvate or succinate, the acidic nature of these polysaccharides is their only apparent common characteristic. The characterization of a new acidic EPS isolated from a saprophytic biovar III strain of *P. fluorescens* (designated H13) obtained from mushroom casing medium [2] is the subject of this report. In contrast to the other EPSs produced by *P. fluorescens*, this polysaccharide contains acetamido sugars, one of which (2-acetamido-2-deoxy-mannuronic acid) has, to our knowledge, been identified in only two other EPS structures [3,4].

2. Experimental

Analytical methods.—Standard procedures were used for determining total carbohydrate [5], amino sugar [6], uronic acid [7] and protein [8]. Monosaccharide composition was determined by hydrolyzing the polymer in 4 M HCl for 2 h at 105 °C. After neutralization with Ag_2CO_3 the samples were converted to alditol acetates and analyzed by GLC on a SP2330 (Supelco) capillary column (15 m), with oven temperature programming from 150 to 250 °C

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[1] Mention of brand or firm names does not constitute an endorsement from the U.S. Department of Agriculture over others of a similar nature not mentioned.

at 4 °C/min. Permethylation analysis was done according to the procedure of Bjorndal et al. [9], with the exception that the permethylated polysaccharide was hydrolyzed with 4 M HCl as above. Mass spectra were obtained on a Hewlett–Packard 6890 GLC system fitted with a Hewlett–Packard 6890 mass selective detector. The absolute configuration of the monosaccharides was determined by the method described by Gerwig et al. [10]. Assignments were established by co-chromatography with sugars of known configuration. The absolute configuration of the uronide was determined after carboxyl reduction.

EPS preparation and purification.—Overnight starter cultures (10 mL per flask) were added to 2800-mL Fernbach flasks, each containing 500 mL of modified King's medium B broth (tryptone substituted for proteose peptone #3) [11] with the glycerol content raised from 1 to 5% (w/v). Cultures were incubated at 20 °C for 5 days with shaking (250 rpm). After incubation, cultures were subjected to homogenization in a commercial blender to separate any capsular EPS present from cells [2]. Cells were then removed from the homogenate by centrifugation (10 000 × g, 15 min), and the EPS was isolated from the supernatant by repeated (3 ×) precipitation with 2-propanol (3 vol). Finally, the EPS was extensively dialyzed against distilled H_2O at 4 °C and lyophilized.

The EPS was subjected to mild hydrolysis with 0.5 M trifluoroacetic acid (TFA) at 70 °C for 90 min. After concentration to dryness the residue was dissolved in 3 mL of H_2O and applied to an Econopak DG-10 (Bio-Rad) gel filtration column (mw cutoff, 10 000). The fraction eluting with the initial 4 mL was designated HMW H13.

Anion-exchange chromatography of the native sample was carried out on a DEAE-Sepharose column. The sample was dissolved in 0.05 M TRIS-HCl buffer (pH 6.5). After washing with buffer the column was eluted with a gradient (0–2 M NaCl) in the same buffer. The polysaccharide eluted between 0.8–1.0 M NaCl.

O-Deacetylation of HMW H13.—The polymer was dissolved in concd NH_4OH, and after standing at room temperature overnight, the solution was concentrated to dryness.

Reduction of HMW H13.—The procedure of Taylor and Conrad [12] was modified as follows. After formation of the activated ester of O-deacetylated HMW H13 with 1-ethyl-3-(3-aminopropyl)carbodiimide, $NaBD_4$ was added to the solution to make it approximately 2 M with respect to borodeuteride; the reaction mixture was then dialyzed for 48 h and the procedure repeated. The dialyzed sample was chromatographed on a DG-10 column, and the void fraction (i.e., that eluting with the first 4 mL) was designated reduced HMW H13.

Partial hydrolysis of HMW H13.—The polymer was hydrolyzed with 1 M HCl at 100 °C for 1 h, neutralized with Ag_2CO_3, and then chromatographed on a Bio-Gel P-2 (fine) column (162 × 1 cm). The fraction with an elution volume approximately equivalent to chitobiose was further purified by preparative HPLC using an Aminex HPX-42A (Bio-Rad) column with H_2O as the eluant (0.4 mL/min) at 65 °C.

NMR analysis.—Spectra were obtained on a Varian Unity + 400 MHz spectrometer at 30 °C. Typically, 2D spectrometer conditions were: 2 K data points; 4 kHz spectral width; 90° pulse (6.8–7.2 μs); 2 s recycle time; 8–32 transients (8 for TOCSY and 32 for HMQC/HMBC experiments). Prior to setting up the 2D experiments, residual water presaturation (SLPSAT) was performed. Once the presaturation was optimized, these identical spectral conditions were utilized with the appropriate TOCSY (TNTOCSY; mixing times of 50, 100, 150 and 300 ms) or HMQC/HMBC experiments. In the HMQC

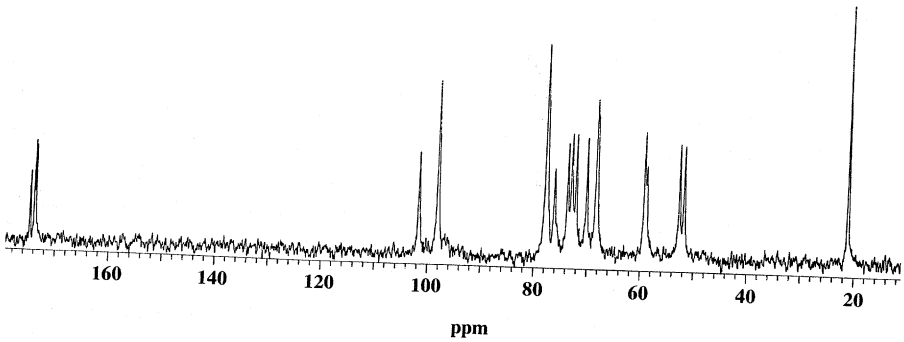


Fig. 1. ^{13}C NMR spectrum of O-deacetylated HMW H13.

experiment, GARP ^{13}C decoupling was applied during the acquisition period. For 1D proton and carbon NMR spectra typical concentrations were approximately 1.0 and 10 mg/mL respectively. For 2D NMR experiments concentrations were 10 to 25 mg/mL.

3. Results and discussion

Aqueous solutions of EPS precipitated from culture filtrates of *P. fluorescens* strain H13 were very viscous at concentrations of 2 mg/mL or greater. The monosaccharide composition of a 2 M TFA hydrolysate (1 h, 120 °C) of this material was determined to be rhamnose, fucose, arabinose, glucose, 2-amino-2-deoxyglucose (or the corresponding acetamide) and an unknown peak eluting after 2-amino-2-deoxyglucose in the gas chromatogram of the alditol acetates. Using strong acid conditions, (4 M HCl, 2 h, 105 °C) glucose and 2-amino-2-deoxyglucose accounted for more than 90% of the sugar composition. An acidic polysaccharide was isolated by anion-exchange chromatography of the crude EPS that contained only minor amounts of rhamnose, fucose and arabinose relative to glucose and 2-amino-2-deoxyglucose. Treating the crude EPS with 0.5 M TFA under relatively mild conditions yielded a much less viscous solution which on gel-filtration chromatography could be fractionated into a high- ($> 10\,000$) and a low-molecular-weight fraction designated HMW H13 and LMW H13, respectively. A yield of about 35% was obtained for HMW H13 which, on sugar (alditol acetate) analysis, was shown to contain glucose, 2-amino-2-deoxyglucose and a minor unknown peak eluting after 2-amino-2-deoxyglucose; the ^{1}H NMR spectra of the ion-exchange purified material and HMW H13 were identical. This result, in conjunction with the fact that the arabinose, fucose and rhamnose were determined to be non-terminal sugars, indicates that the deoxyhexoses and arabinose are constituents of a co-precipitating polymer(s) and not associated with the glucose and 2-amino-2-deoxyglucose containing polysaccharide.

Signals at 52.1, 53.5, 21.2, 173.9 and 173.7 ppm in the ^{13}C NMR spectrum of O-deacetylated HMW H13 (Fig. 1) indicated that the repeating unit contains two acetamido sugars. The total number of carbon resonances (22) and the number of carbon resonances observed in the anomeric region (3) of the ^{13}C NMR spectrum obtained without NOE indicated that the polymer is composed of a trisaccharide repeating unit

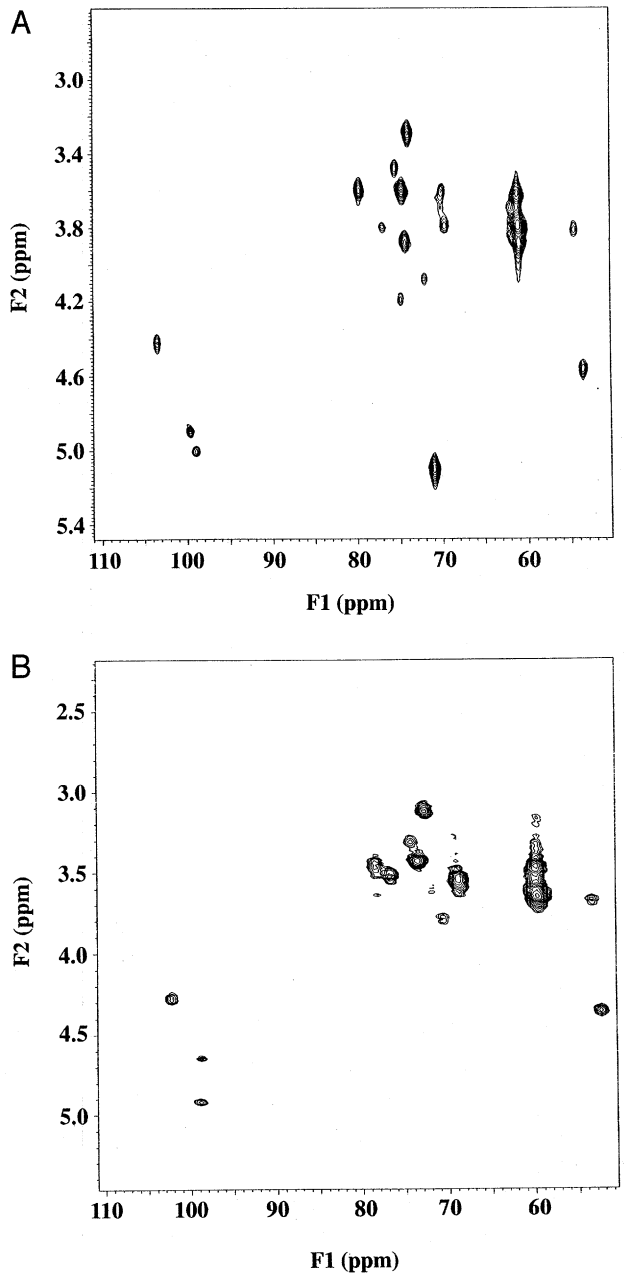


Fig. 2. (a) $^{1}H-^{13}C$ correlation map of HMW H13. (b) $^{1}H-^{13}C$ correlation map of deacetylated HMW H13.

with two acetamido groups. Although the HMW H13 fraction was retained on an anion-exchange chromatography column, and a peak was observed in the ^{13}C NMR spectrum at 174.7 ppm, colorimetric assay for uronic acid was negative. HMW H13 reduced by the Taylor–Conrad method [12] using $NaBD_4$ was shown to contain by GLC–MS of the alditol acetates besides glucose and 2-amino-2-deoxyglucose, 2-amino-2-deoxymannose dideuterated at C-6, which must have resulted from the reduction of 2-acetamido-2-deoxy-mannuronic acid. The retention

Table 1
^{1}H and ^{13}C NMR spectral assignments for acetylated HMW H13

Residue		Chemical shifts						
		C-1	C-2	C-3	C-4	C-5	C-6	C-6′
4)-β-Glc	^{1}H	4.32	3.34	3.77	3.76	3.64	3.94	4.01
	^{13}C	102.5	73.0	73.5	79.0	74.0	60.5	
4)-α-GlcNAc	^{1}H	4.92	3.76	4.02	3.53	3.71	3.92	3.68
	^{13}C	98.5	53.5	71.0	78.9	76.0	60.5	
3)-β-ManNAcA	^{1}H	4.81	4.48	3.78	5.0	4.11	–	–
	^{13}C	99.0	52.1	77.5	70.5	72.0	174.5	

Table 2
^{1}H and ^{13}C NMR spectral assignments for O-deacetylated HMW H13

Residue		Chemical shifts (ppm)						
		C-1	C-2	C-3	C-4	C-5	C-6	C-6′
4)-β-Glc	^{1}H	4.38	3.22	3.54	3.76		3.64	3.50
(J_{C1-H1} 167 Hz)	^{13}C	101.80	72.10	73.00	77.90	na [a]	59.80	
(J_{H1-H2} 7 Hz)								
4)-α-GlcNAc	^{1}H	4.92	3.68	3.80	3.60	3.83	4.00	
(J_{C1-H1} 180 Hz)	^{13}C	98.60	53.00	70.20	77.90	na	59.80	
(J_{H1-H2} < 1 Hz)								
3)-β-ManNAcA	^{1}H	4.65	4.35	3.73	3.65	na		
(J_{C1-H1} 165 Hz)	^{13}C	98.20	52.10	77.90	68.40	na	174.7	

[a] Not assigned.

time of the aminomannuronic acid derivative corresponded to that of the unknown peak observed in the sugar analysis of HMW H13. The presence of small amounts of the 2-amino-2-deoxymannose derivative in the original sugar analysis most likely resulted from the reduction of lactone that was formed during the preparation of the alditol acetates. The HMQC spectrum (Fig. 2a) confirmed that the repeating unit is a trisaccharide. The HMQC also indicated that the polysaccharide is O-acetylated (δ_H, 5.04 ppm; δ_C, 70.5 ppm). These assignments were confirmed by comparison with the HMQC of the base-treated (deacetylated) polymer (Fig. 2b). The resonance at δ_H, 4.35 ppm in the ^{1}H NMR spectrum, is clearly the H-2 of one of the 2-amino-2-deoxy sugars (δ_C, 52.1 ppm). On the basis of NMR COSY, TOCSY and HMQC spectra (Table 1) and permethylation results (Table 3), the trisaccharide is composed of 4-substituted β-glucose, 4-substituted 2-acetamido-2-deoxy-α-glucose and 3-substituted 2-acetamido-2-deoxy-β-mannuronic acid, all in the pyranose form.

The sequence of the repeating unit was determined by an HMBC experiment on the O-deacetylated HMW H13. If glucose were substituted on the *N*-acetyl mannuronic acid, then a coupling of C-1 of the glucose (103.3 ppm) to H-3 of the 2-acetamido-2-deoxymannuronic acid (3.73 ppm) would be apparent; for the other possible sequence in which the glucose is substituted on the 2-acetamido-2-deoxyglucose, a coupling between the C-1 of glucose (103.3 ppm) and H-4 of the 2-acetamido-2-deoxyglucose (3.60 ppm) would be observed. The latter was the observed coupling. A disaccharide was isolated from a partial hydrolysate of carboxyl-reduced HMW H13, which on subsequent reduction, followed by hydrolysis and then acetylation, yielded acetylated glucosamine and 2-aminomannitol, which is consistent with the HMBC data.

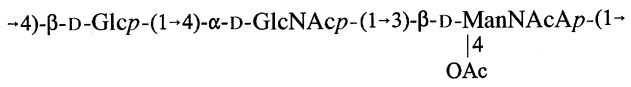
The location of the O-acetyl substituent was also apparent by comparing the NMR data for the acetylated (Table 1) and O-deacetylated (Tables 2 and 3)

Table 3
Permethylation analysis

Residue	HMW H13	Reduced HMW H13
4)-Glc	+	+
4)-GlcN	+	+
3)-ManN	–	+

HMW H13. A significant upfield shift was observed for H-4 of the 2-acetamido-2-deoxymannuronic acid when the EPS was O-deacetylated (H-4, native polymer, 5.0 ppm; O-deacetylated polymer, 3.65 ppm).

Absolute configurations were determined [10] on the hydrolyzed reduced HMW H13, and the complete structure of the repeating unit of the EPS is determined to be as follows:



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